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AGGLUTININS AND LYSINS IN THE MOLLUSCAN FAMILY PLANORBIDAE: A SURVEY OF HEMOLYMPH, EGG-MASSES, AND ALBUMEN-GLAND EXTRACTS

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Substances which agglutinate or lyse human and other mammalian erythrocytes occur naturally in the hemolymph and in extracts from various tissues of molluscs. The function of these substances has not been determined; however, Prokop and his associates (1968a, b) assume that they play a role in the defense mechanisms of gastropods and coined the term "protectin". Pemberton (1974) noted that agglutinins have been detected in nearly 80 species of gastropods, including some species of Planorbidae (Boyd, Brown, and Boyd, 1966; Brown, Almodovar, Bhatia, and Boyd, 1968; Gilbertson and Etges, 1967; Lee-Potter, 1969; Pemberton, 1971; Rudolph, 1973).

Gilbertson and Etges (1967) observed differences in the distribution of hemolymph hemagglutinins in species and strains of *Biomphalaria* and suggested that these substances might be of value in differentiating populations of planorbids. In view of the limited number of strains employed in their study, Gilbertson and Etges' suggestion requires further evaluation. In fact, Pemberton (1974, p. 104) reviewed the literature on variation in terrestrial gastropods and concluded that, "There is, however, no current evidence that agglutinin content and specificity are of taxonomic value at species level."

In the present study, an effort was made to assess both interspecific and interpopulation differences in members of the Family Planorbidae with respect to the presence and specificity of agglutinins and lysins. Extracts of albumen glands and egg-masses, as well as hemolymph, were tested from 31 strains representing 8 species and 4 genera of Planorbidae.

Materials and Methods

Snails were reared and maintained at 26 ± 1° C in 1.5, 5, or 10 gallon aquaria and fed romaine lettuce. The water was aerated and filtered, and the snails were exposed to fluorescent light for 12 hr daily. Species and strains of snails, their geographic origins and date of collection are listed in Table I.

Preparation of samples

Snail hemolymph samples were obtained by methods previously described (Michelson, 1966), and each sample represented a pool of hemolymph from 4–8 snails (12–15 mm in diameter).

To prepare egg extracts, egg-masses were removed from sheets of plastic film which had been placed previously in stock aquaria. No effort was made to separate

the masses with respect to age or stage of development. The masses were rinsed in distilled water, blotted on paper toweling, and weighed on a micro-balance. They were then placed in a tissue-grinder and 5.5 μl of 0.85% saline added for each milligram of egg-mass. The contents were homogenized by hand for approximately 5 min, then centrifuged at 300 g for 5 min, and the supernate used as the test-sample.

To prepare albumen-gland extracts, albumen glands were removed from 5–10 mature snails (12–15 mm in diameter), weighed, and homogenized in a tissue-grinder containing 25 μl of 0.85% saline per milligram of tissue. The homogenate was centrifuged at 300 $\times$ g for 5 min, and the supernate used as the test-sample.

Hemagglutination tests

Serial doubling dilutions of test-material were prepared in U-bottom wells of Microtiter® plates (Cooke Engineering Co., Alexandria, Virginia). Dilutions were made in 0.85% phosphate-buffered saline (pH 7.2) at a final volume of 0.25 ml per well. An equal volume of a 2% human red-blood cell suspension was then added, the plates were covered, mixed on a mechanical shaker, and allowed to sit undisturbed for one hr at 27° C or overnight at 4° C. Control wells contained only the diluent and red-blood cells. Unless otherwise noted, a minimum of three different samples of each extract or hemolymph was tested for every snail strain. Test samples were used within one hour of preparation.

Studies, comparing agglutinins and lysins contained in the hemolymph and extracts from various species and strains of Planorbidae, utilized A_1, B, and O erythrocytes from the same series of three donors. To test for the possible variability in the receptor-cells, forty different samples of erythrocytes were obtained from either members of the Department of Tropical Public Health or from the Transfusion Center of the Children's Medical Center, Boston, Massachusetts. This collection of erythrocytes contained samples from twelve A_1, four A_2, three A_1B, six B, and fifteen O-type donors. The cells were subsequently used in tests employing albumen-gland extracts from seven strains of *B. glabrata*.

Since hemolymph and tissue extracts were pooled from series of snails of the various strains in the survey study, it was necessary to determine the degree of variability that might occur among individuals of a particular strain. Consequently, hemolymph from twenty-nine snails of the S-3 strain of *B. glabrata* and from ten snails of the CB strain of *H. caribaeum* was individually tested against A_1-type cells obtained from a single donor. In addition, extracts of individual albumen glands from ten snails of the S-3 strain were tested against A_1-type cells from a single donor.

Characteristics of hemolymph and tissue extracts

A preliminary and limited effort was made to characterize the physicochemical properties of the agglutinins contained in hemolymph and tissue extracts. Thus, albumen-gland extracts and egg extracts from two strains of *B. glabrata* (B-1 and S-3) were subjected to heat inactivation at 56° C and 80° C for one hr; dialysis against TrisHCL buffer (pH 7.8) for 24 hr; freezing at −20° C for up

TABLE 1

Geographic origins of snail species and strains.

Species and strains	Origin and date of colonization
Biomphalaria glabrata	
PR-1	Puerto Rico, March 1954
PR-2	Rio de la Plata, Puerto Rico, December 1957
PR-2′	Strain derived from single egg of a PR-2 snail, April 1959
PR/B*	Puerto Rican/Brazilian hybrid, March 1966
B-1	Catinga do Moura, Bahia, Brazil, June 1964
B-2	Salvador, Bahia, Brazil, June 1964
S-3	Lake Amaralina, Salvador, Brazil, July 1964
BH*	Belo Horizonte, Minas Gerias, Brazil, July 1964
G-1	Fazenda Graviel, Castro Alves, Bahia, Brazil, June 1974
G-2	Fazenda Graviel, Castro Alves, Bahia, Brazil, June 1974
G-3	Fazenda Graviel, Castro Alves, Bahia, Brazil, June 1974
M-1	Fazenda Morro do Afonso, Castro Alves, Bahia, Brazil, June 1974
RS-1	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
RS-2	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
RS-3	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
RS-8	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
St.L.	Cul de Sac Valley, Castries, St. Lucia, March 1969
Biomphalaria straminea	Recife, Pernambuco, Brazil, May 1964
Helisoma caribaeum	
257	Capella Viaja, Puerto Rico, July 1959
CH	San Juan, Puerto Rico, December 1970
DB	Dos Bocas, Puerto Rico, July 1959
CB	Castle Burke, St. Croix, Virgin Islands, August 1959
B	Bogota, Colombia, December 1970
Helisoma anceps	
H′ (pigmented and albino)	(?) Strain originated from tropical fish aquarium, September 1954
GB	Grand Bahama Island, June 1970
Bulinus truncatus	
E-1	Egypt, June 1957
E-2	Liberian Institute (? Egypt), December 1963
Bulinus globosus	
N-1	Nigeria, August 1972
SA-1*	South Africa, November 1965
Polypylis hemisphaerula	Liu Ying, Tainen, Taiwan, June 1963

* Albino strain.

to one yr; and repeated freezing and thawing for ten consecutive times. Extracts so treated were titered against A_1 erythrocytes and compared to freshly prepared extracts. In addition, attempts were made to block the agglutinating activity of albumen-gland extracts from B-1 and PR-2′ strains of *B. glabrata* by incorporating 0.1 M concentrations of various sugars in 0.85% saline in hemagglutination tests employing A_1 cells. Sugars used in this series of tests were as follows: N-Acetyl-D-Galactosamine, α-D(+)Fucose, L(−)Fucose, D(+)Galactose, β-D(−)Fructose, α-D(+)Glucose, α-Lactose, D(+)Maltose, D(+)Mannose, L(−)Sorbose, and Sucrose. These sugars were used also in attempts to block the agglutination of A_1, B, and O erythrocytes by hemolymph from *H. caribaeum* (CB).

Results

Survey of strains and species

Egg extracts. Agglutinins for one type or another of human erythrocytes were detected in egg extracts from all seventeen strains of *B. glabrata* (Table II). The specificity of the reactions allowed a grouping of the strains into two categories: those whose agglutinins reacted only against A-type cells (B-2, S-3, BH, G-3, St.L), and those whose agglutinins reacted against both A- and B-type cells. The type of reaction observed did not appear to be correlated with albinism. Undiluted samples from strains B-1 and RS-2 agglutinated O-type cells; however, for practical purposes, reactions initiated by undiluted samples may be ignored. *B. straminea* agglutinated only A-type cells.

Except for extracts from the albino varient of *H. anceps* (H′) and from the E-2 strain of *B. truncatus*, in which undiluted samples agglutinated A-type cells and A- and B-type cells, respectively, agglutinins were not detected in the samples from the other strains and species.

Albumen-gland extracts. Albumen-gland extracts were prepared from all geographic strains of *B. glabrata* except the G-1 strain. Again, the reactions permitted the grouping of snails into two categories (Table III); those which react solely with A-type cells and those which react with both A- and B-type cells. The PR-1 strain showed slight reaction (1 : 4) in some samples, but not all, with O-type cells. Concentrated extracts of two strains gave evidence of lysis, but extracts of others did not.

Table II

Agglutinins detected in extracts of egg-masses from Biomphalaria *strains and species.*

Species and strains	RBC specificity and maximum titer		
	A_1	B	O
Biomphalaria glabrata			
PR-1	16	32	0
PR-2	64	64	0
PR-2′	32	64	0
PR/B	32	8	0
B-1	256	64	1
B-2	32	0	0
S-3	64	0	0
BH	64	0	0
G-1	8	16	0
G-2	8	16	0
G-3	16	0	0
M-1	16	16	0
RS-1	16	16	0
RS-2	8	8	1
RS-3	16	32	0
RS-8	16	32	0
St.L.	8	0	0
Biomphalaria straminea	64	0	0

TABLE III

Agglutinins detected in extracts of albumen-glands from strains of Biomphalaria glabrata.

Strains	RBC specificity and maximum titer		
	A_1	B	O
PR-1	32	32	4
PR-2	16	16	nd*
PR-2′	16	16	0
PR/B	32	1	0
B-1	16	4	0
B-2	32	0	0
S-3	128	0	0
BH	32	0	0
G-1	nd	nd	nd
G-2	16	16	0
G-3	16	4	0
M-1	8	32	0
RS-1	8*	8	*
RS-2	8	16	0
RS-3	8	8	0
RS-8	4	8**	nd
St.L.	64	1	0

* nd = not done.
** Signifies lysis in undiluted sample.

Extracts were prepared from three strains of *H. caribaeum* (257, CB, B), but no agglutinins were detected. The small size (4–5 mm) of *Polypylis* precluded the use of albumen-gland extracts and a whole snail extract was used. All tests were negative.

Hemolymph. Agglutinins were detected in the hemolymph from only six strains of *B. glabrata* (S-3, BH, G-2, G-3, M-1, B-2), and all had titers less than 1:8 except for the S-3 strain (1:32). A-type cells only were agglutinated by hemolymph from strains S-3, BH, and B-2; whereas, hemloymph from strains G-2, G-3, M-1 was reactive only against O-type cells. The S-3 strain gave higher titers at 4° C than at 27° C; titers for other strains were higher at 27° C. It is of interest that no Puerto Rican strain exhibited agglutinins, an observation first noted by Gilbertson and Etges (1967). Lysins were found in the hemolymph of all strains at either 4° or 27° C. Titers were generally low and rarely exceeded 1:2 in tests conducted at 27° C and only up to 1:16 in samples tested at 4° C. The lysins were nonspecific and reacted with all cell types.

Agglutinins in high titer were detected in six strains of *Helisoma* (Table IV). Strain 257 of *H. caribaeum* had titers up to 1:2048, the highest observed in any freshwater snail and possibly the highest hemolymph titer for any snail studied thus far. The agglutinins, however, showed no specificity and reacted with all types of cells. Lysins were observed in all strains except CH, B, and GB, and their titers were slightly higher than those of *Biomphalaria.*

In the genus *Bulinus,* only a South African strain of *B. globosus* gave evidence of having agglutinins. The titers were low and never exceeded 1:4.

TABLE IV

Agglutinins in the hemolymph of Helisoma *species and strains (27° C).*

Species and strains	RBC specificity and maximum titer		
	A_1	B	O
Helisoma caribaeum			
257	2048	256	256
DB	64	32	8
CH	0	0	0
CB	256	256	32
B	512	256	32
Helisoma anceps			
H′ (pigmented)	16	16	16
H′ (albino)	32	16	8
GB	0	0	0

Variability of receptor cells

Some variability was detected in the response (titer) of albumen-gland extracts when tested against cells obtained from a variety of donors (Table V) and those from the pools used in the survey of species and strains; however, strains in both studies reacted in a similar manner, and the results of both studies were comparable. Those strains that were reactive to A cells alone remained so, as did those which previously showed reactivity to both A- and B-type cells. No reactions were noted with O-type cells at titers greater than 1 : 1. Although the number of A_1B and A_2 donors was limited, it was observed from the material available that extracts from all strains agglutinated A_1B cells at titers from 1 : 8 to 1 : 64. A_2-type cells were either not agglutinated or reacted only at very low titers, usually less than 1 : 4.

Variability of extracts and hemolymph from individual snails

Although differences in titer were observed among hemolymph samples from individuals of CB strain of *H. caribaeum* and in extracts of the albumen-glands from individual S-3 snails, all snails in both groups responded uniformly in demonstrating the presence of agglutinins. In the *Helisoma* samples, all tests showed titers of 1 : 256, which was as far as the material was diluted. Titers from the albumen-glands, on the other hand, ranged from 1 : 8 to 1 : 128 with a geometric mean titer of 1 : 45.3. Hemolymph samples from S-3 snails were highly variable, some samples showing the presence of agglutinins (up to 1 : 64), whereas others were negative.

Preliminary characterization of agglutinins

Neither heat inactivation at 56° C, dialysis, nor repeated freezing and thawing appeared to effect the agglutinating activity of egg or albumen-gland extracts. Extracts frozen for up to three months at −20° C showed no loss of reactivity and those frozen for one year only showed a loss of one titer.

A remarkable difference was noted in the response of *Helisoma* hemolymph and

Biomphalaria albumen-gland extract to the presence of various sugars in the test system. N-Acetyl-D-Galactosamine effectively blocked agglutination of A_1-, B-, and O-type cells by *Helisoma* hemolymph, whereas the other sugars had no effect. On the other hand, N-Acetyl-D-Galactosamine had no effect on albumen-gland extracts from strains of *B. glabrata.* Albumen-gland extracts were markedly inhibited by α-D(+)Fucose, α-L(−)Fucose, β-D(−)Fructose, D(+)Mannose, L(−)Sorbose, D(+)Maltose, α-D(+)Glucose, and Sucrose. Partial inhibition was exerted by D(+)Galactose and α-Lactose.

Discussion

Hemagglutinins and hemolysins for human erythrocytes are not uniformly present in members of the family Planorbidae, and differences were observed between genera and species as well as between populations of a single species. The ability to detect these substances depends, in part, on the selection of the sample to be tested, and variations were noted between hemolymph, albumen-gland extracts, and extracts of egg-masses. Thus, lysins were found primarily in hemolymph samples, rarely in albumen-gland extracts, and never in egg-mass extracts. When hemolymph served as the test sample, agglutinins were detected in only 6 of the 17 *B. glabrata* populations; however, all populations exhibited agglutinins in egg and albumen-gland extracts. If one assumes that the albumen-gland is the source of agglutinins and that these substances are sequestered into developing eggs, as well as disseminated into the surrounding hemolymph, then this apparently erratic distribution can be explained. Failure to identify agglutinins in the hemolymph of some populations could be due to "spill-over" concentrations too low to detect

Table V

Agglutinins detected in extracts of albumen-glands from strains of Biomphalaria glabrata: *summary of trials employing RBC's from various donors.*

Strains	RBC specificity and mean titer*		
	A_1	B	O
BH	27.8 (10)**	0 (6)	0 (15)
	8–64	0–1	
PR/B	26.5 (11)	5.0 (6)	0 (15)
	4–128	2–8	
S-3	46.7 (11)	0 (6)	0 (13)
	16–256	0–2	
PR-2′	20.7 (8)	12.7 (6)	0 (15)
	8–128	4–32	
G-2	8.7 (8)	12.7 (6)	0 (15)
	4–32	4–32	0–2
B-2	47.8 (11)	0 (4)	0 (12)
	8–256	0–1	0–1
B-1	19.0 (8)	5.7 (6)	0 (15)
	8–64	2–32	0–1

* Geometric mean and range.

** Numbers in brackets equals number of donors tested.

by the techniques employed. On the other hand, agglutinins were not found in either egg or albumen-gland extracts of any of the species of *Helisoma*. However, agglutinins were detected in the hemolymph of some strains and species and at very high titers. This suggests that the origin of the agglutinins in *Helisoma* may be different from those of *Biomphalaria*. The lack of specificity of the hemolymph agglutinins also distinguishes them from those of *Biomphalaria*. Moreover, when the results of the inhibition studies are considered, differences between the agglutinins of the two genera become patently apparent; N-Acetyl-D-Galactosamine effectively blocks *Helisoma* agglutinins, but has no effect on those from *Biomphalaria*. Likewise, those sugars which inhibited *Biomphalaria* agglutinins failed to be effective against agglutinins from *Helisoma*.

Although the limited number of species and strains of *Bulinus* and *Polypylis* tested prevents a definitive evaluation of these genera, it appears likely that both groups lack agglutinins specific for human erythrocytes. Brown *et al.* (1968) noted some reactivity with extracts from *Bulinus truncatus* when enzyme modified cells were employed, and the hemolymph of some *Bulinus* species appear capable of agglutinating nonhuman mammalian cells (Rudolph, 1973).

Gilbertson and Etges (1967) reported that the agglutinins found in the hemolymph of *Biomphalaria* lack specificity and reacted identically against A, B, and O-type cells. Our data appears to contradict theirs in that reactions occurred with either A or O-type cells, but not with both and not at all with B-type cells to the exclusion of A. These differences are not easily explained, since Gilbertson and Etges' snail strains from Bahia, Brazil were derived from our S-3 and B-2 colonies. Techniques differed in the type of hemoagglutination test employed, but this alone does not necessarily account for the differences observed. It may be suggested that the observed differences are due to variability of the erythrocytes obtained from different donors, but this does not agree with our experience. It should be noted, that the present study indicated that *Biomphalaria* hemolymph is a poor source of agglutinins, extremely variable, and not suitable alone for comparing species and strains.

The present study lends support to the thesis that lectins (agglutinins and lysins) may be a valuable tool in characterizing snail populations and aiding in the taxonomic discrimination of freshwater snail species. When egg or albumen-gland extracts were employed as a source of agglutinins, populations of *B. glabrata* could be segregated into two major groups—those which reacted with A-type cells and those that reacted with both A- and B-type cells. Further characterization could be made employing data on hemolymph lysins and agglutinins. The applicability for this purpose of nonhuman erythrocytes and enzyme altered cells in the test-system remains to be explored and may contribute to further differentiation.

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Summary

1. Thirty-one strains representing eight species and four genera of the molluscan family Planorbidae were surveyed for the presence of hemagglutinins and hemolysins. Extracts prepared from albumen-glands and egg-masses, as well as hemolymph, were assayed by a micro-hemagglutination technique in which human erythrocytes were used as receptors.

2. Hemagglutinins and hemolysins for human erythrocytes were not uniformily present in all members of the family and detection of these substances depended, in part, on the material tested.

3. Neither heating to 56° C, dialysis, storage at −20° C for up to a year, nor repeated freezing and thawing appeared to effect the agglutinating activity of egg or albumen-gland extracts.

4. Inhibition of the agglutinating activity of *Helisoma* hemolymph could be accomplished with N-Acetyl-D-Galactosamine, but this sugar had no effect on the activity of *Biomphalaria* agglutinins.

5. There is evidence to suggest that the source and nature of agglutinins from *Helisoma* and *Biomphalaria* species are different.

6. Lectins may be of some value in characterizing snail populations and as an aid in the taxonomic discrimination of species.

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